BIOCHEMICAL ASPECTS OF GENETICS[1]

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It would be impossible, in the time and space available, to cover all of the noteworthy advances that have been made in biochemical genetics during the past year. We have, therefore, selected for review a number of topics that are especially active at the moment and that promise to yield important new results in the near future. At the same time, we have tried to avoid duplicating the material of other chapters in this volume which are germane to biochemical genetics. We refer, in particular, to the chapters on *Metabolism of Nucleic Acids* (*Macromolecular DNA and RNA*); *Nucleic Acids and Protein Biosynthesis; The Basic Proteins of Cell Nuclei;* and *Chemistry of Differentiation in Lower Organisms*. The reader should consult these reviews, as well as the present one, for a fuller view of current activities in this field.

By way of introduction, we should like to call attention to some papers of general interest. The formation of animal viruses in bacterial cells infected with viral DNA has been reported from two laboratories. Abel & Trautner (1) first showed that DNA prepared from vaccinia virus is capable of infecting *Bacillus subtilis* and giving rise to complete virus particles. This result was confirmed by Bayreuther & Romig (2), who found that DNA from polyoma virus also infects *B. subtilis*, with the production of complete virus. Since, in both cases, formation of virus particles involves the synthesis of serologically specific coat proteins, these results constitute compelling evidence for universality of the genetic code. A curious sidelight to these findings has been the rediscovery of a series of papers published in the 'thirties by Silber and co-workers in Moscow, in which the growth of pox, herpes, and other animal viruses in yeast and bacteria was described [see (3, 4) and subsequent papers in the same journal].[2]

A fundamental advance has been made simultaneously by Yanofsky et al. (5), working with the A protein of *E. coli* tryptophan synthetase, and by Sarabhai et al. (6), with the head protein of bacteriophage T4D. Both groups of workers proved colinearity of the gene with its cognate protein.

Roberts (7) has studied an unusual series of mutants in *Aspergillus*. The genes control utilization of sorbitol and are unusual in that they show complementation in *trans*-heterozygous diploids but not in heterocaryons. The

[1] The literature survey for this review was completed in October 1964.
[2] We are indebted to Dr. K. E. Bayreuther for these references.

same effect has been noted occasionally in other fungi, but this case provides a particularly good opportunity to investigate what may be a new mode of genic interaction. It is now established that interallelic complementation in heterocaryons results from interaction of protein subunits in the cytoplasm, but the nature of the interaction is not understood. A useful discussion of this problem has been given by Crick & Orgel (8). Morrow (9) has examined the heterocaryon method for measuring the frequency of irreparable lethal mutations in *Neurospora*. He finds that, as predicted (10), this method overestimates this frequency owing to interactions between the mutant gene and the marker genes present in the heterocaryon. Metzenberg, Kappy & Parson (11) have found that seven out of sixteen independent ethionine-resistant mutants of *Neurospora* are irreparable lethals or sublethals at 38°. One of the mutants was selected for study and was shown to overproduce methionine. This result suggests that derepression of methionine synthesis accompanies some irreparable lethal mutations and that repression control of amino acid pathways resides in indispensable macromolecules.

Repression and Induction in Procaryotes[3]

A mathematical treatment of regulatory systems has recently been published by Dean & Hinshelwood (12). Their model is based on the supposition that a change in the concentration of any intermediate in the cell will result in a change in the concentration of every other intermediate. The equations of state are soluble for steady-state conditions; but, according to the authors, the method runs into mathematical difficulties in the analysis of transient responses. This model may become very useful and have great predictive value when a sufficient number of parameters can be measured to assign values to the coefficients. It seems less likely that the Dean-Hinshelwood formulation will lend itself to exploring the molecular mechanism of regulation.

The simple but elegant model of control that has emerged to a large extent from the work of Jacob & Monod (13) received immediate and almost universal acceptance. Because the large amount of evidence supporting this theory has been the subject of a number of highly articulate reviews (14–17), only some of the most recent corroborations will be reported below. As even the most welcome theory deserves criticism, which in this instance has not always been prominent, some aspects of the model which remain to be clarified will be emphasized.

One of the most important predictions of the operon theory is that repression is controlled at the level of transcription of genetic information at the genome and, that for any given operon, a given messenger or group of messengers will be present in a cell at a higher steady-state level when the cell is derepressed with respect to the relevant pathway than when it is repressed (or genetically deleted). Martin (18) used two *Salmonella typhimurium*

[3] In the interests of continuity, a few examples from the eucaryotes *Neurospora* and *Saccharomyces* have been included in the discussion of procaryotes.

strains, one of which was constitutive for histidine synthesis and the other of which contained a long deletion in the histidine operon. By using double labelling, he was able to identify a 34S messenger RNA from the derepressed cells. The molecular weight of this species of RNA corresponded remarkably well with that predicted for a single messenger capable of coding for all of the proteins of the histidine operon, assuming a coding ratio of three.

In a similar vein, Attardi and co-workers (19) showed that induction of the pathway of galactose utilization in *E. coli* by fucose produced a large increase in the amount of a specific RNA—namely, that RNA which is incapable of hybridizing with DNA not carrying the genes for galactose utilization (λDNA), but which can be hybridized with DNA in which this region is present (λ*dg* DNA). By this technique, it was possible to achieve a great improvement in the signal-to-noise ratio. As might be predicted, the RNA corresponding to the galactose message was present in galactose operator-constitutive (O^c) *E. coli*, even in the absence of inducer. In a related study, messenger corresponding to the lactose operon was detected by hybridizing DNA from episomes carrying the *Lac* region with the appropriate RNA preparations after first cross-absorbing the latter with DNA obtained from bacteria which carried a deletion in the *Lac* operon. It was also shown that operator-negative (O^0) mutants failed to produce significant amounts of the specific RNA corresponding to the *Lac* operon either in the presence or absence of inducer. This fact is somewhat surprising. Beckwith (20) has shown that O^0 mutants are suppressible. According to current theories on the mode of action of unlinked suppressors, the transcription process in suppressible mutants should be unimpaired.

Guttman & Novick (21) have also described the detection of messenger RNA corresponding to the *Lac* operon by a method similar to that employed by Martin (18) for the histidine operon. Possibly message production is tightly coupled to message use.

Hayashi et al. (22) detected messenger, corresponding to the *Lac* region of *E. coli*, in a comparable manner by using a source of DNA for hybridization which was highly enriched with respect to *Lac* operon—namely, a phage which specifically carries the *Lac* region. Once again, the noise level was reduced by methods analogous to those used by Attardi et al. (19).

With these independent and almost simultaneous findings, that regulation of the rate of synthesis of a given protein is correlated with the presence of a discrete fraction of RNA, the theory seems well established, at least in Enterobacteria. Whether this conclusion can be safely extended to more complex organisms remains a moot point (23) and will be discussed in a later section of this review.

An additional prediction implicit in the operon model in its unadorned form is that all proteins whose structural information devolves from a single operon will be formed in equimolar amounts under all conditions; this is clearly not the case. Nishi & Zabin (24) have shown that the ratio of β-galactosidase to thiogalactoside transacetylase, both of which are controlled by

the same operator, varies considerably at different temperatures. Additional evidence has been presented by Zabin (25) that, under all conditions, the molar equivalent of transacetylase present in the cell is much smaller than that of the β-galactosidase. Similarly, Ames & Hartman (26) have reported preliminary evidence supporting the notion that the various enzymes specified by the histidine operon of *Salmonella* are synthesized in differing molar quantities. In each case, a protein specified by a cistron close to the operator gene is synthesized in larger molar amounts than one which is located more distally with respect to the operator. On an even firmer basis is the general finding (13) that mutations which decrease or eliminate one enzyme may cause a proportional decrease in all enzymes which are specified by cistrons distal (but never proximal) to the mutation. This phenomenon, termed "polarity," has been attributed to a decreasing probability of translation of a given messenger. The segment of RNA transcribed from cistrons nearest to the operator would have the greatest likelihood of being successfully translated into protein. Ames & Hartman (26) and Stent (14) have discussed the possibility that there is a number of "modulating triplets" at critical points in any messenger-RNA molecules which are complementary to the anticodons of "rare" transfer-RNA molecules. If a ribosome encounters a triplet for which no transfer RNA is immediately available, it might become disengaged from the messenger and fail to translate all distal portions of the messenger. Thus, the probability of translation of a given segment would be decreased by the compounded jeopardy of all the preceding modulating triplets. For a more detailed discussion of modulation and polarity, the reader is enthusiastically referred to the above reviews.

One older observation on that most orthodox of control systems, the lactose operon, seems inconsistent with the concepts that have been presented above. Pardee (27) has noted that galactinol and a number of other compounds are effective as inducers of the galactoside permease, but are ineffective, or relatively so, as inducers of β-galactosidase. As the structural gene for β-galactosidase is situated between that of the permease and the operator, compounds such as galactinol appear to be affecting translation of the messenger in an antipolar fashion.

Lee & Englesberg (28) have made the equally baffling observation that mutations in the structural gene for the ribulokinase of *E. coli* affect the levels of both arabinose isomerase and of ribulose-5-phosphate-4-epimerase; the structural genes for these two enzymes lie on either side of that for ribulokinase. Furthermore, mutations in the ribulokinase gene can either increase or decrease the levels of the isomerase and epimerase; but, whichever is the case, the latter enzymes are always present in a fixed ratio, i.e., are coordinately controlled. Evidence is available that these enzymes are qualitatively identical with those of the wild-type strain. The amounts of protein corresponding to ribulokinase in the various mutants could not be assessed enzymatically because of the structural changes in this protein. However, such immunological estimations of the inactive ribulokinase as could be

made suggested that the levels of this protein are also affected coordinately with the other enzymes. Hence, "modulations" in the arabinose operon can affect cistrons in both directions and can increase or decrease the final expression of these cistrons. Lee & Englesberg point out that mutations in the structural gene for β-galactosidase which produce greatly increased levels of the galactoside permease might be adversely affected by the high intracellular level of an unmetabolizable sugar (lactose); *ergo*, lethal; *ergo*, undetected. This explanation seems somewhat unlikely for two interdependent reasons. Firstly, gratuitous inducers such as *iso*propyl thiogalactoside, which are not hydrolyzed by β-galactosidase, can be accumulated to high levels without deleterious effects. Secondly, it might be expected that increased levels of permease would have more effect on the rate of uptake than on the final steady-state intracellular level. However, it remains possible that mutations analogous to those studied by Lee & Englesberg might be lethal in both the lactose and the histidine systems.

The Nature of Repressor Substances

Despite intensive study of cellular control mechanisms, the repressor remains a logical construct rather than a chemical entity. As the most economical variant of the operon model suggests that the repressor be produced directly by the chromosome itself, it seemed logical that the repressor substance be a specific RNA molecule (13). Some earlier work tended to support this conclusion by demonstrating that the repressor of the lactose operon could be synthesized in merozygotes, even in the presence of 5-methyl tryptophan or chloramphenicol, which inhibit protein synthesis; hence, the repressor could not be a protein and must be some other information-carrying macromolecule, presumably RNA. The latter studies also suggested that the repressor is synthesized relatively slowly, viz., the accumulation of a biologically significant concentration of repressor in a cell requires a period of time equal to about half the normal doubling time of the cell. This finding is at odds with results obtained with the control system for *E. coli* alkaline phosphatase, as will be discussed below.

Returning to purely theoretical considerations, various authors have reasoned that the repressor substances must be endowed with considerable specificity in order to recognize, unambiguously, the various low molecular-weight effectors. Such specificity has long been recognized as a property of the active sites of enzymes and, more recently, of the "nonactive" or allosteric sites of many proteins. Smith et al. have noted, however, that the specificity of recognition of effectors by repressor substances may be more permissive than the recognition of effectors by enzymes which are subject to end-product inhibition (29). In addition, the finding that the *E. coli* repressor of alkaline phosphatase synthesis is furnished as such by the taxonomically distant organism *Serratia marcesens* (30) suggests that the interactions of repressors with their sites of action are also not of the highest order of specificity.

If one holds that the repressor substances are proteins rather than nucleic acids, it becomes much easier to see how these substances can specifically bind various effectors. Simultaneously, it becomes more difficult to visualize the manner in which the repressor substance, with or without effector, can be bound to a specific DNA segment (the operator), because proteins and amino acids are, in general, not known to be capable of highly specific interactions with nucleic acids (31). However, at least one limited exception to this generalization can be provided. Clearly, there is specific recognition between a given activating enzyme and its repertoire of cognate acceptor RNA molecules. Apparently, methylating enzymes also recognize sequences in nucleic acid. Hence, it is quite possible that the perplexity raised by the hypothesis that repressor molecules are proteins is more apparent than real.

In at least one case, it is definitely known that a protein is intimately involved in a regulatory system, although the nature of this involvement is by no means clear. Garen & Otsuji (32) have isolated and characterized a protein which is produced by the *R-2* gene, one of two loci which regulate the synthesis of alkaline phosphatase in *E. coli* (33). This protein is missing from extracts of some, but not all, *R-2*-constitutive mutants. Among the constitutive strains which are able to synthesize this protein, some are "nonsense mutants" as judged by the fact that they are suppressible (i.e., the strain is rendered repressible) by the presence of a nonallelic mutation. This mutation presumably allows the translation of an otherwise meaningless codon of messenger RNA. It would be expected, then, that the unsuppressed nonsense mutants at the *R-2* locus would be unable to synthesize the *R-2* protein. The authors circumvented this apparent contradiction by providing some genetic evidence that the *R-2* locus actually consists of two cistrons: the *R-2a* cistron specifies the protein which was being investigated, and the *R-2b* cistron specifies an unidentified protein and contains all suppressible mutations which do not impair the synthesis of the *R-2a* protein.

The most surprising feature of the system is that the synthesis of the *R-2a* protein is repressed under almost exactly the same conditions as those which cause the repression of alkaline phosphatase itself. Hence, when the *R-2a* protein is absent for genetic reasons, the synthesis of alkaline phosphatase is constitutive; when the *R-2a* protein is at low levels for nutritional reasons (viz., repletion with inorganic phosphate), the synthesis of alkaline phosphatase is repressed. It seems clear from this and other evidence that the *R-2a* protein is not, itself, the repressor substance, nor is it a lineal precursor of the latter. More likely, it is an enzyme, perhaps a phosphorylase, which is required for the formation of the elusive repressor substance. In the absence of inorganic phosphate, the *R-2a* protein would accumulate in the cell to high levels; but repression would not occur because the effector, inorganic phosphate, is absent. In the presence of the latter, the *R-2a* protein would be diluted out by cell division until a low, but apparently sufficient, steady-state level is reached. If one assumes that the reaction of effector with repressor is

at least bimolecular, it becomes clear that such a system would be very responsive to sudden changes in the levels of inorganic phosphate.

A number of workers have studied temperature-sensitive repression systems. Gallant & Stapleton (34, 35) have described a mutation of the *R-2* control gene for alkaline phosphatase which allows repression to occur at low temperatures but causes semiconstitutive synthesis of phosphatase at elevated temperatures. Apparently, the rate of synthesis of the functional phosphatase repressor is reduced in this strain, and much more drastically so at higher temperatures. The reduced rate of synthesis allowed a kinetic analysis of the reappearance of repression under various conditions in cells which had previously been depleted of repressor by phosphate starvation. Such an analysis would be technically very difficult with wild-type cells in which the resynthesis of repressor is apparently very rapid. Chloramphenicol treatment of such derepressed cells greatly decreased the rate of resynthesis of repressor. Canavanine treatment, which in *E. coli* inhibits arginine synthesis and hence protein synthesis, was even more effective than was chloramphenicol. While these findings do not prove that the repressor itself is a protein, they do indicate that protein synthesis is required for the re-establishment of repression. It seems doubtful that the regulatory substance affected in this temperature-conditional repression system is the *R-2a* protein of Garen & Otsuji; the *R-2a* protein is present only in the absence of inorganic phosphate and is metabolically stable.

Novick, Lennox & Jacob (36) have also described a system in which the synthesis of a repressor substance is temperature-sensitive. The authors studied the kinetics of escape of growing cells from the repressed state following a sudden increase in the temperature. It was shown that the loss in repression, which was reflected in this case by the gradual appearance of β-galactosidase, occurred approximately equally in all cells and was not an all-or-none response by a small proportion of the cell population. Thus, there must be a finite affinity of the repressor for the operator, and a stoichiometric equality between these elements would not cause total repression. If one assumes, with the authors, that at elevated temperatures the repressor is stable and is diluted out only by bacterial growth and division, it can be deduced from the kinetic data that the reaction between repressor and operator may be of a higher order than bimolecular. For alternative interpretations, the reader is referred to the original paper.

Horiuchi, Horiuchi & Novick (37) have isolated a mutant in which the repressor itself is unusually thermolabile, rather than the repressor-synthesizing system. The temperature sensitivity of this altered repressor substance is consistent with the possibility that the latter may be a protein.

The evidence presented to date does not seem to allow a decision as to whether repressor substances are proteins or some other type of macromolecule. Pardee & Prestidge (38) have shown that the repressor of the *Lac* operon of *E. coli* can be made in the presence of inhibitors of protein synthesis. Recent evidence of Sypherd & DeMoss (39) has established that re-

pressors of both lactose utilization and of tryptophan synthesis are preferentially formed by exponentially growing cultures in the presence of low levels of chloramphenicol. The latter authors were able to rule out catabolite repression as the basis for repression of the lactose system, and they excluded tryptophan accumulation as the grounds for repression of tryptophan synthetase. The repressor of ornithine transcarbamylase, studied by Bowne & Rogers (40), appears to fall into the same category.

On the other hand, Gallant & Stapleton's demonstration (35) that chloramphenicol preferentially inhibits the formation of the repressor of alkaline phosphatase must be reconciled with the above findings. It is possible to argue that all repressors are proteins: the synthesis of some repressors being inhibited more strongly by chloramphenicol than is bulk protein synthesis, the synthesis of others being unusually resistant to this inhibitor. However, it seems possible that some repressors are proteins, while others are not. Occam's Razor may become a dangerous instrument when elevated from a working hypothesis to a law of nature.

There is reason to believe that the repression of enzymes involved in the synthesis of an amino acid may be effected not by the amino acid itself, but by aminoacyl-sRNA. Schlesinger & Magasanik (41) have found that α-methylhistidine can cause derepression of the enzymes of histidine biosynthesis in *E. coli*. As shown earlier by Moyed (42), thiazolealanine can cause derepression of these enzymes as an epiphenomenon of end-product inhibition of the first enzyme in the sequence. This inhibition leads to temporary histidine starvation which, in turn, leads to derepression. However, α-methylhistidine does not cause significant end-product inhibition of the first, nor of any other enzyme in the pathway. The authors showed that the latter analogue, which is not incorporated into protein, decreases the rate of synthesis of histidyl-sRNA *in vitro*. The analogue does not itself become attached to sRNA, nor is it a substrate for the activating enzyme, as judged by the pyrophosphate exchange reaction. It was suggested, therefore, that the analogue directly inhibits the transfer of histidine from its adenylate complex with the activating enzyme to the cognate sRNA. The data do not seem to exclude the possibility that α-methylhistidine may compete with histidine for an active site on the enzyme without itself being a substrate. Although the authors interpret their results cautiously, the evidence appears strong that histidyl-sRNA is at least more closely related to the active repressor than is free histidine. If aminoacyl-sRNA species are, in fact, the active and complete repressors of amino acid biosyntheses, it is unlikely that all, or even most, of the many and varied repression systems can be similarly explained in a credible and esthetically satisfying way.

Some very interesting observations relevant to the control of RNA synthesis have been reported. Earlier work by Borek, Rockenbach & Ryan (43) had resulted in the isolation of a mutant of *E. coli* which, unlike wild type, continued to synthesize RNA for some time after being deprived of an amino acid for which it had a nutritional requirement. This mutant, which may be

isolated as a prototrophic recombinant, has been termed a "relaxed" strain as opposed to the "stringent" wild-type strain, the alleles being designated as RC^{rel} and RC^{st}, respectively. Much fruitful work has been made possible by the isolation of this strain [Neidhardt & Eidlic (44); Borek (45)]. Recently, Alföldi et al. (46) noted that when RC^{rel} cells are transferred from an amino acid-rich medium to a minimal one, the adaptation of the bacteria to growth on minimal medium is achieved much more slowly than is the case with wild type. Addition of certain individual amino acids, especially leucine, to the minimal medium greatly aggravates the physiological impasse, such that most of the cells never escape from repression of amino acid synthesis. As the authors point out, cells which have been shifted from a rich medium to a minimal one are, temporarily, "physiological auxotrophs." It is, therefore, not surprising that the RC^{rel} strain, unlike wild type, continues to synthesize RNA after such a shift. An interpretation suggested by the authors is as follows. A series of repressors of RNA synthesis exist, any one of which is sufficient to halt RNA synthesis. Each of the repressors is normally neutralized by a specific amino acid. The RC^{rel} strain may contain a "catholic inducer" which is absent or ineffective in wild type and which is able to neutralize the various repressors in a nonspecific manner. Under conditions which would cause the catholic inducer itself to be in short supply (e.g., growth on a rate-limiting carbon source), the mutant would behave in a manner similar to wild type, which is, indeed, the case. For reasons that can only be surmised, the continuation of bulk RNA synthesis during amino acid starvation makes the transition to growth on minimal medium much more difficult. Possibly the continuation of ribosomal and transfer-RNA synthesis in the RC^{rel} strain competes with necessary messenger synthesis to the detriment of the cell.

Catabolite repression.—It has become increasingly evident that catabolite repression, which has classically but too restrictively been referred to as "the glucose effect," constitutes a control system which is epistatic to the types of induction-repression which have been discussed above. McFall & Mandelstam (47) have pointed out that dual-control systems could be expected to confer a competitive advantage upon an organism. For example, a strain of *E. coli* which possessed only the repressor system specified by the *i* gene would unnecessarily synthesize β-galactosidase when presented with a mixture of glucose and lactose; conversely, a strain having only catabolite repressibility would needlessly produce this enzyme when grown on any rate-limiting carbon sources, including those which are not substrates for the enzyme. A natural example of the latter sort of non-goal-directed behavior can be cited. In *Neurospora*, invertase is produced most abundantly with galactose as sole carbon source, a condition which allows relatively slow growth. Sucrose, which, via invertase itself, is an excellent carbon source, evokes relatively little invertase production [Metzenberg (48)].

Experiments by Neidhardt & Magasanik (49) and by Mandelstam (50) have shown that the repressive effects usually attributed to glucose are best

explained by the fact that catabolism of glucose is unusually rapid relative to cell growth, as compared with other carbon sources, and causes the accumulation of large pools of various metabolites. Mandelstam's experiments, which employed a continuous growth apparatus, show that if growth is limited by some nutrient other than the carbon source (e.g., the nitrogen source) any compound capable of serving as a carbon source will mimic the effects of glucose and will severely repress the synthesis of β-galactosidase. If the carbon source is withheld completely, the nongrowing cells readily synthesize the enzyme by turnover of pre-existing protein.

The distinction between catabolite repression of β-galactosidase synthesis and the more familiar *i*-gene-operator gene system is clearly pointed out by the finding of Brown (51) that lactose operator-constitutive mutants are still subject to catabolite repression. Mandelstam (50) has reported that i^--constitutive cells are likewise catabolite-sensitive, and that catabolite repression of these cells is completely unaffected by inducer. Loomis & Magasanik (52) reasoned that even the i^- allele might produce a material which is active in the presence of catabolites; however, they were able to exclude this possibility by conjugation experiments in which an i^+z^+ chromosome was introduced into a female carrying a deletion of the entire lactose region, including the *i* gene. The authors found that the synthesis of β-galactosidase in the newly formed merozygotes was immediately subject to catabolite repression, whereas the accumulation of the product of the *i* gene is known to occur quite slowly under these conditions.

The finding that catabolite repression is a phenomenon distinct from other repression systems poses a question as to the stage in protein synthesis at which it acts. Nakada & Magasanik (53) have presented evidence that it operates at the level of messenger synthesis. On the other hand, the findings of Hauge at al. (54) suggest that glucose repression of the β-glucosidase of *Saccharomyces* may occur at the level of release from the ribosome. It is, of course, possible that both of these modes of operation play a role.

Catabolite repression of various systems cannot be attributed to the same catabolite in each case. Neidhardt & Magasanik (55) showed that, under appropriate conditions, the repression of histidase by glucose can be lifted without a general escape of the cells from catabolite repression. McFall & Mandelstam (56) have shown that pyruvate is most efficient in the repression of tryptophanase and D-serine deaminase, whereas galactose is most effective in repressing β-galactosidase synthesis. In each case, the most effective compound is a product of the enzyme in question. The latter authors have found that galactose exerts this repressor effect even in a series of mutants which lack either the kinase, transferase, or epimerase enzymes of galactose utilization. The epimeraseless mutants, however, were relatively insensitive to glucose repression. It seems to the reviewers that, if the strain deficient in the kinase turns out to be totally devoid of this enzyme and no other pathway of galactose assimilation is present, then galactose per se is quite likely an effector of catabolite repression.

Finally, it is possible that catabolite repression and the more familiar re-

pression mechanisms have much more in common than is evident from studies of the lactose system. McFall (57) has obtained one-step mutations in which both the inducibility and the catabolite sensitivity of D-serine deaminase synthesis are affected.

Recent evidence indicates that even in the case of repressed genes, a round of transcription may occur at the time of replication of DNA. Hanawalt & Wax (58) synchronized thymine-requiring *E. coli* cells by adding thymine to previously starved cultures and showed that the total β-galactosidase content of an uninduced culture doubled relatively abruptly some 20 to 35 min following the addition of thymine, after which the absolute rate of synthesis again decreased. In this manner, the basal or uninduced level of the enzyme was normally maintained constant at one or two molecules per cell. In the absence of thymine, basal synthesis of the enzyme stopped immediately, although protein synthesis continued for some time. By contrast, and in harmony with the results of Nakada (59), the induced synthesis of β-galactosidase proceeded even in the absence of DNA synthesis and paralleled gross protein synthesis. The authors present evidence that the basal synthesis of alkaline phosphatase (in the presence of excess inorganic phosphate) likewise requires DNA synthesis.

If the assumption is made that the uninduced β-galactosidase is present in approximately equal amounts in each cell, a quantitative check may be performed. Kiho & Rich (60) have estimated that between three and ten polyribosomes bearing the message of the *Lac* operon are present in each fully induced cell. As the rate of synthesis of β-galactosidase in such cells is about 1000-fold higher than the basal rate, it might be expected that the basal level would be maintained if 0.003 to 0.010 polyribosomes were continuously present. Less formalistically, this corresponds to one polyribosome being present for only 0.3 to 1.0 percent of a cell generation, or about 0.18 to 0.60 minute's unimpaired usage of the messenger. This should be compared with the half-life of β-galactosidase messenger, which has been estimated as 1 to 2.5 min by Kepes (61) and Nakada & Magasanik (53), and corresponds to several minutes of unimpaired usage. Perhaps messengers are less stable in certain phases of the cell cycle.

Gorman et al. (62) showed that in a haploid strain of *Saccharomyces* dividing synchronously, β-glucosidase was synthesized at a single definite time in the division cycle and that this behavior persisted through a number of synchronous divisions. When a hybrid diploid yeast was similarly examined, it was found that two distinct bursts of β-glucosidase synthesis occurred during each division cycle. In a diploid strain containing a single functional gene for α-glucosidase and two functional genes for invertase, one and two bursts of synthesis of the respective enzymes were observed during each cycle. Hence, the phenomenon can probably be considered a general one. A crucial difference between this system and that of Hanawalt & Wax should be noted. In the yeast system, even induced synthesis is periodic and occurs at the same phase of the cell cycle as does basal synthesis [Halvorson et al. (63)].

The possibility that at least some genes are transcribed only at the time of replication allows some interesting speculations on the control of cell division. For example, if some element necessary for the next round of replication (e.g., DNA polymerase) were transcribed only during replication, then the act of replication itself would be required to prime the cell for the next act of replication. This could furnish a mechanism by which otherwise identical populations of cells could either divide repeatedly, or remain in the resting state indefinitely, if the required element were somehow lost. Whether such a model will be relevant to development, tumorigenesis, etc., remains to be seen.

The Promoter

Jacob, Ullman & Monod (64) have recently isolated a large number of lactose operator-constitutive (O^c) mutants from an i^+o^+ deletion $Fi^+o^+z^+y^+$ strain. Such O^c mutations comprised a high proportion of the mutants which arose either spontaneously, or as a result of X-ray treatment, or somewhat less so by exposure to ethylmethane sulfonate, and were absent among the constitutive mutants which were induced by ultraviolet light or 2-aminopurine. This fact, taken with the observation that no revertible O^c mutants were found, suggests that all of them are deletions. Although the levels of constitutive β-galactosidase synthesis varied from 2 percent to 100 percent of that of the fully induced wild type, all of the O^c strains could be induced to the 100 percent level. Furthermore, the inactivation kinetics of the β-galactosidase from a number of the strains were found to be indistinguishable under a number of conditions from those of the wild-type enzyme. Hence it is probable that O^c mutants, unlike O^0 mutants, are the result of a change which is purely regulatory and are not "extreme polarity mutants" which reside in the z gene. Beckwith (65) has shown that deletion of the first part of the z gene affects neither the gross ability of the rest of the operon to be transcribed nor its sensitivity to regulation. As the O^c mutants have lost part or all of their sensitivity to regulation, it was postulated (*a*) that there must be an initiation point governing expression of the operon, either at the level of transcription or of translation (Jacob et al. favor the former view); and (*b*) that this initiation point, termed "the promoter," lies between the regions defined by the O^c and the z^- mutations. The recognition site for the repressor is in the operator region.

Using a homogenote diploid for the super-repressor gene i^s, the product of which appeared to have lost its affinity for inducers, a large number of O^c mutants were isolated, of which about 2 percent had the genotype $O^cz^-y^+$. Of these, all had deletions which included the i and the O^c regions as well as various lengths of the z gene. By the nature of the selective method, it was assured that the y gene could be expressed in all of the strains. Tests of ten of the strains showed that the galactoside transacetylase was also present in amounts up to 40 percent that of the fully induced wild type and that its levels were completely insensitive to inducer. Thus, it seems likely that the hypothetical promoter is necessary for the expression of the operon only if the

i region is intact. A provisional explanation can be offered: each operon contains an upper-case letter or promoter at the initiation of reading, and a period at the end as well. Destruction of the promoter alone would prevent expression of the operon. Simultaneous destruction of the period following the preceding operon, which in this case might reside in or near the *i* region, would place the entire operon under the control of the operator gene of the preceding operon, with concomitant loss of the normal regulatory mechanism (66). This interpretation is not totally without difficulties. For example, the still-intact segment of the *Lac* operon would probably be attached somewhere near the distal end of its unknown neighbor operon, and would thus be subject to most, if not all, of the "downward" modulations which would presumably exist in the expression of the neighboring operon. Since no special steps were (or could be) taken to induce or derepress this neighbor, it follows that the basal rate of transcription and translation of the neighboring operon must be sufficient, after all modulations, to allow at least 40 percent of the maximum possible rate of synthesis of the succeeding segments of the *Lac* operon. In view of the fact that probably most of the genes in any organism are concerned with quantitatively minor pathways, it seems surprising, although of course not impossible, that the *Lac* operon should have for its nearest neighbor an operon which not only governs another quantitatively major function but is also normally derepressed.

Repression, Induction, and Differentiation in Eucaryotes

Repression and induction obviously have great survival value to procaryotes in their struggle with their protean environment and with one another. These mechanisms must have evolved as a result of selective pressures in which the unit of survival is the single cell and the criterion of success is cell division. Regulatory mechanisms are surely of importance to differentiated organisms as well. However, the nature of the selective pressures that have guided their evolution are probably very different; a cell from the intestinal mucosa would gain no long-term advantage by successfully invading the ecos that is ordinarily occupied by the kidney. In addition, most of the cells in many eucaryotes (e.g., mammals) are maintained in a relatively constant chemical environment. This is accomplished to a large extent by the liver, which, by virtue of its portal circulation, is rather directly exposed to the variable environment of the intestinal lumen and functions as a chemostat. It is hardly surprising that the liver is the site of almost all of the induction-repression systems that have been described in higher animals.

In view of the differing selective pressures that operate on various organisms, it seems well possible that the resulting mechanisms of regulation in eucaryotes and procaryotes might be quite dissimilar. For instance, in bacteria, the genetic representation of an entire metabolic pathway is very frequently in the form of a unit of transcription, i.e., an operon; by contrast, the genes corresponding to a given metabolic pathway in eucaryotes are usually scattered, more or less randomly, over the genome. One of many available examples of this difference is seen in the enzymes of histidine biosynthesis.

In *Salmonella*, these are present in a single operon (26). In *Neurospora* [Haas et al. (67), Ahmed, Case & Giles (68)] and in yeast (69), the genes are widely dispersed, although in each case there is one chromosomal region that appears to specify three different enzymatic reactions. This region might correspond to a greatly truncated operon. The organizational differences between *Salmonella* and the eucaryotes are made more impressive by Fink's report that the metabolic pathways are identical in the three organisms.

A single example is available in which an operon in yeast corresponds reasonably closely to one that has been studied in *E. coli*. Douglas & Hawthorne (70) have discussed ten different loci that are concerned with galactose utilization in *Saccharomyces*. Of these, three are very tightly linked and specify the kinase, transferase, and epimerase enzymes. The remaining seven loci are weakly linked or unlinked to the "operon" and to one another. One of the unlinked genes is concerned with the permease, one with phosphoglucomutase, and one with long-term adaptation to galactose. An unlinked gene concerned with inducibility has been identified; mutations at this locus cause a galactose-constitutive phenotype which, as expected, is recessive to the wild-type allele. The term "operon" must be used guardedly. No mutants that are truly analogous to either O^0 or O^c mutants have been found in the yeast system. Instead, mutations in one of the unlinked genes have the remarkable property of preventing the expression of all genes in the "operon." Strains carrying a mutation of this sort are not comparable to the super-repressor or the O^0 mutants of *E. coli* [Willson et al. (71)], as the yeast mutants are unlinked to either the "operon" or the inducibility locus, and are subject to complementation.

In a recent report, Horowitz, Fling & Asano (72) have noted that in *Neurospora*, tyrosinase and L-amino acid oxidase are normally undetectable during vegetative growth, but can be simultaneously derepressed by starvation after a lag period of 9 to 12 hr, or can be induced by certain inhibitors of protein synthesis in a medium which otherwise does not allow synthesis of these enzymes. This situation is reminiscent of the induction of alkaline phosphatase in *E. coli* by canavanine and chloramphenicol (35), and suggests that the repressor is a protein. Tyrosinase is ordinarily present in *Neurospora* during the sexual phase [Horowitz et al. (73)], which is induced by growth in a nitrogen-limited medium. A control mutant, *ty-1*, fails to produce either tyrosinase or L-amino acid oxidase during starvation, unless it is also induced by an inhibitor of protein synthesis. Furthermore, this strain is female-sterile. It seems likely that these biochemical and morphological responses are controlled, perhaps coordinately, by a single repressor, which in *ty-1* is not readily inactivated during starvation. It is not known whether the structural genes for tyrosinase and L-amino acid oxidase are closely linked, as in an operon. No mutants with a structural alteration in the latter enzyme have as yet been isolated, and hence the gene remains unmapped.

Perhaps the ultimate in the genetic dispersion of functionally related entities is the situation in which two different polypeptide chains of a given

protein are specified by cistrons which are located on different chromosomes. Gross (74) found this to be the case with the β-carboxy-β-hydroxyisocaproate isomerase of *Neurospora*. Likewise, DeMoss (75), also working with *Neurospora*, has reported that two unlinked loci contribute polypeptide chains to anthranilate synthetase. In the classic example of nonidentical subunits, human hemoglobin, it appears that the genes carrying the information for the α and the β chains are not closely linked [Smith & Torbert (76); Itano, Singer & Robinson (77)]. In none of these cases is there reason to believe that the synthesis of the two complementary chains is normally uncoordinated. The existence of hemoglobin H, which contains four β chains [Jones et al. (78)], suggests that such a situation is not categorically impossible, but that the regulatory mechanisms usually keep the synthesis of the necessary chains closely coordinated.

Since it seems clear that the organization of genes into extensive operons is not an absolute requisite for control of metabolism, it is fitting to ask what selective pressure has produced or maintained the integrity of operons in procaryotes. Horowitz (79) has discussed the possibility that the emergence of operons is an inevitable consequence of the evolution of metabolic pathways by gene duplication followed by gene modification. He has pointed out the difficulties involved in subsequently separating such genes by chromosomal rearrangements, especially in organisms in which sexual or parasexual events are rare. An alternative speculation is that the process of transduction, which is not prominent in eucaryotes, is of considerable survival value to procaryotes, provided that an entire metabolic pathway can be acquired in a single transductional event. To satisfy this proviso, the functionally related cistrons would have to be extremely closely linked. Even if this is the case, it is somewhat difficult to visualize the nature of the selective process; presumably it would not be the donor bacterium on which the selective advantage would be conferred. In coenocytic organisms such as *Neurospora*, the phenomenon of heterocaryosis may play a major role in the metabolic coordination of sequential enzymes. Those hyphal tips which contain an ideal ratio of various nuclei with quantitatively different capacities would presumably be selected during growth of the mycelium.

In the light of our current understanding of regulatory processes at the molecular level, two fundamental questions should be posed: (*a*) is the selective and time-specific transcription of various elements of the genome to messenger RNA highly meaningful in the processes of development and differentiation; and (*b*) is it sufficient in itself, such that all other phenomena can be relegated to a status of secondary and derivative (albeit complex) interactions? An attempt will be made to show that the probable answers to these questions are (*a*), yes; and (*b*), no. In considering the dependence of any developmental event upon the level of a given messenger RNA, it is probably useful to recall that the initial messenger concept called for a species of RNA which is metabolically very unstable. This concept was soon amended to include more stable messages, such as those for the synthesis of

hemoglobin in reticulocytes; and the term "long-lived messenger RNA" has become common. The obvious fact should be noted that when messenger of an indefinitely long half-life must be postulated, the concept of a "messenger" becomes heuristically rather useless, as "messenger" cannot then be distinguished from other stable components of a cell.

Much of the evidence that the synthesis of new messengers is essential for any given alteration in higher organisms rests on the fact that the alteration in question is inhibited by actinomycin D. While the evidence that this antibiotic can act by blocking transcription of DNA is beyond challenge [Reich (80)], it is much less certain that it has no other mode of action whatsoever [Revel, Hiatt & Revel (81)]. Moreover, actinomycin D inhibits all DNA-directed RNA synthesis, including ribosomal and transfer RNA; and messenger RNA of rat liver may not even be the most sensitive of these three functional types [Harel et al. (82)]. Rosen et al. (83) have reported that when actinomycin D is administered to rats in limited amounts over a period of days, a number of enzymes (among them, tryptophan pyrrolase) actually increase in amount both in intact and adrenalectomized animals [for other comparable findings, see (83)]. This effect is almost certainly an indirect one, secondary to the inhibition of some as yet unidentified system. However, it provides warning that the congruent (and less easily detected) situation may exist—namely, that even where the synthesis of new RNA of the messenger sort can be shown to be required for a given regulatory or developmental event, it does not necessarily follow that messenger RNA synthesis is the prime mover in that event. Somewhat similarly, Garren et al. (84) have reported data which, if interpreted in the least subtle manner, would be taken as evidence for the induction of tryptophan pyrrolase and tyrosine transaminase by actinomycin D. For a more reasonable interpretation, the reader is referred to the original article. Conversely, in a process that is insensitive to actinomycin D, the lack of dependence on messenger-RNA synthesis can be proved with complete rigor only if it can be shown that the entire genome is accessible to the antibiotic. In spite of these caveats, data obtained by use of actinomycin D have been of inestimable value, and can be interpreted with fewer reservations than would be the case with many other commonly used inhibitors.

In addition to the inherent problems of studying control processes in highly complex organisms in which "biochemical" mutants are relatively rare, and genetic analysis is usually prohibitively tedious, other impediments to rapid progress exist. Even in *Neurospora*, the range of specific activities over which an enzyme can be caused to vary is much less than that encountered in bacteria—generally of the order tenfold rather than 1000-fold. Donachie (84a), for example, finds that the formation of aspartate carbamoyl transferase is limited to a repression-derepression variation of only twofold to threefold in *Neurospora*, in contrast to the 1000-fold variation found in *E. coli* for the same enzyme. An exception to this generalization has been provided by Pitot & Peraino (85), who noted a 300-fold increase in the hepatic

threonine dehydrase of rats following intubation of casein hydrolysate. The puromycin sensitivity of this induction strongly suggests that protein synthesis, rather than activation, is involved. In addition, when actinomycin D was administered at the same time as the casein hydrolysate, induction was completely inhibited, which implies that the induction was conditional upon the synthesis of the new RNA. If administration of actinomycin D was delayed for 12 hr, there was little inhibition of the continuing synthesis of enzyme. Hence, the messenger for synthesis of threonine dehydrase is probably formed during the early phase of the induction and, once formed, is reasonably stable.

During metamorphosis of tadpoles of *Rana catesbeiana*, the levels of enzymes of the ornithine cycle rise sharply in the liver. Brown, Brown & Cohen (86) showed that carbamyl phosphate synthetase, argininosuccinate synthetase, argininosuccinase, and arginase undergo increases of the order of 20-fold in specific activity during development of a premetamorphic tadpole into an adult frog, and that the increases occur simultaneously and in a quasicoordinate manner. Ornithine transcarbamylase seems somewhat discoordinated from the other enzymes in that it rises only 8.6-fold during the same period and parallels the others less closely with respect to time of increase. Paik & Cohen (87) found that thyroxin induces a precocious metamorphosis of the ornithine cycle enzymes, as well as anatomical development. Furthermore, the enzymatic changes occur more rapidly than the morphological changes with which they are correlated during *ad libitum* metamorphosis, so that creatures are obtained which are biochemically frogs, but anatomically still tadpole-like.

The data of Schimke (88) on induction of enzymes of the ornithine cycle in rats can be profitably compared with those from tadpoles. A high-protein diet brings about large increases of these enzymes in the liver. In this instance, all of the enzymes increase more or less simultaneously except arginase, which requires a somewhat longer time to reach its maximum value. Further studies by the same author have demonstrated that in several lines of HeLa cells (which fail to convert ornithine to citrulline, but can convert citrulline to arginine) synthesis of the two enzymes required for the latter process was repressed by high levels of arginine, whereas arginase was induced to higher levels by this treatment (89). Thus, arginase and the arginine-synthesizing enzymes are capable of being controlled in an anti-parallel, as well as a parallel, fashion. The arginine-synthesizing enzymes in liver have as their major function the detoxification of ammonia via urea production; in HeLa cells, which are derived from a carcinoma of the human *cervix uteri*, these enzymes almost certainly are oriented toward the anabolism of arginine for protein synthesis. The differing control systems for arginase and the arginine synthetase enzymes in liver and in HeLa cells call to mind the acetolactate synthetases of *Aerobacter aerogenes*. Halpern & Umbarger (90) characterized two highly dissimilar and separately controlled enzymes; both of these formally catalyze the same reaction, but one serves in an anabolic and

the other in a catabolic capacity. On the other hand, it is quite possible that only single molecular species of arginase and of the arginine-synthesizing enzymes exist in any given animal and that, in different tissues, these enzymes may be controlled in entirely different ways. It should be noted that data which deal with the induction of enzymes in higher animals must be interpreted with more sophistication than seems to be required in considering induced synthesis in growing bacterial cultures. In the latter, enzymes are generally metabolically stable, whereas in mammals, the levels of a number of enzymes [including arginase (91)] reflect a balance between synthesis and degradation. Furthermore, the rates of degradation as well as the rates of synthesis can be influenced by the availability of substrate, certain metal ions, etc.

One of the most thoroughly studied enzymes that is inducible in chordate liver is tryptophan pyrrolase [Knox & Mehler (92)]. Recently it has been found by Greengard, Smith & Acs (93) that, while increases in this enzyme can be elicited by either tryptophan or by cortisone and its congeners, only the hormone-induced increase is sensitive to actinomycin D, whereas both types of induction are sensitive to puromycin. It seems probable that the hormone stimulates or derepresses the synthesis of the relevant messenger RNA, and that substrate induction increases the efficiency of expression of pre-existing messengers, mainly or entirely by protecting existing enzyme from inactivation rather than by enhancing its synthesis [Schimke, Sweeney & Berlin (94)].

The response of tryptophan pyrrolase to hormones and to tryptophan is by no means ubiquitous among chordates, nor is it invariant during the development of a single species. Nemeth (95), who has studied several species of mammals, has observed that fetal liver, which lacks this enzyme, cannot be induced to produce it by either substrate or adrenal corticoids. More recently, Spiegel & Spiegel (96) have noted that this enzyme is absent in embryos of *R. catesbeiana*. In early larval stages, the enzyme has an activity comparable with that of adult frogs but decreases to negligible levels during either natural or thyroxin-induced metamorphosis. Higher levels of tryptophan pyrrolase can be obtained by tryptophan administration during those stages of development in which the enzyme is present even without tryptophan administration; during stages wherein it is absent, it cannot be induced. Hormonal induction cannot be demonstrated at any stage. The authors point out that it is difficult to be certain that tryptophan actually reaches its potential site of action during all phases of development, and that it is equally arbitrary to assume that the activity in young tadpoles is attributable to the same polypeptide as is responsible for the activity in adults. However, if one temporarily sets these reservations aside, it appears that the activation and inactivation of the segment of the genome controlling this enzyme is a reversible process which occurs normally during development. The presumption that such reversible processes occur has been widely accepted, but examples which support it, even provisionally, are few. The

changes in the inducibility by tryptophan can probably best be explained by assuming that in frogs, as in rats (94), the fundamental effect of the substrate is the stabilization of existing enzyme.

The fact that hormones play a part in the development of higher animals and plants needs no documentation here. It may be asked whether hormones exert their effects by stimulating the synthesis of certain messenger-RNA species. The evidence cited above suggests that this is the case in the stimulation of synthesis of tryptophan pyrrolase by corticosteroids. Studies of certain other enzymes also support the hypothesis that corticosteroids exert their action through messenger RNA [e.g., Kenney & Kull (97)]. The administration of actinomycin D interferes with at least some of the responses to a number of other hormones, e.g., estrogens, insulin, aldosterone, ecdysone, thyroxin, and parathyroid hormone. However, in most cases, it appears that a given hormone also has other characteristic actions which do not entail new RNA synthesis [e.g., see Tashjian, Ontjes & Goodfriend (98); Rasmussen, Arnaud & Hawker (99); Sokoloff, Francis & Campbell (100)]. Furthermore, such agents as epinephrine, glucagon, and luteinizing hormone act upon their target tissues by indirectly activating phosphorylase [Rall & Sutherland (101); Marsh & Savard (102)], a process which almost certainly does not require the intervention of messenger RNA synthesis.

The case of insulin is typical of hormones that seem to exhibit two or more modes of action. Salas, Viñuela & Sols (103) first showed that a specific glucokinase of liver is greatly reduced in diabetic animals, that it can be induced to normal levels by administration of insulin, and that this induction is inhibited by actinomycin D. The insulin-mediated increase of liver glycogen synthesis was found to be similarly sensitive to the antibiotic. Sövik (104), who has extended the findings concerning glycogen synthesis to rat diaphragm, noted that the insulin-mediated uptake of sugars is insensitive to actinomycin D. Gellhorn & Benjamin (105) have recently shown that the insulin-dependent repair of several other enzyme systems in diabetic animals is completely abolished by actinomycin D, but they have corroborated the finding that the latter antibiotic has no effect on the hypoglycemic action of insulin, i.e., on the cellular uptake of glucose. To make matters more complicated, reports of the direct action of insulin, *in vitro*, upon various enzymes continue to appear [Vester & Reino (106); Ilyin & Titova (107)]. It is quite possible that insulin has a variety of effects which do not radiate from any single fundamental event; on the other hand, it is conceivable that insulin does have some as yet unidentified unitary action. If the latter is the case, this unitary action is almost surely independent of genetic transcription. A frankly speculative possibility for unitary action of certain hormones is offered below.

Recently, considerable evidence has accumulated concerning the mode of action of estrogens on the uterus of ovarectomized rats. Ui & Mueller (108) have found that estrogens stimulate the synthesis of bulk protein and phospholipids, an effect which is prominent about 4 hr after administration of the

hormone. This increased synthesis is blocked by actinomycin D. In a companion article, Noteboom & Gorski (109) reported a still earlier increase (about 2 hr after hormone injection) of RNA polymerase, which is likewise abolished by the antibiotic. Hamilton (110) detected a very small increase in protein synthesis at a time corresponding to the increase in RNA polymerase and roughly simultaneous with the onset of increase in RNA synthesis. He suggested that the increase in RNA polymerase may set the stage for a much larger subsequent increase in protein synthesis. These data, taken together, allow some flights of fancy about the mechanism of action of hormones in general. One can postulate that the fundamental effect of a hormone is to increase the permeability of one or more types of target cell to some ubiquitous nutrient which, at least for that cell, limits overall metabolism (e.g., the effect of insulin on glucose uptake). The ability to act as a target cell would be determined by the developmental history of that type of cell. As a result of a general increase in metabolic processes, the synthesis of a variety of macromolecules, among them RNA polymerase, would be accelerated. Here it should be noted that RNA polymerase, unlike most cellular constituents, might be expected to be under positive feedback control, i.e., autocatalytic. Therefore, the accumulation of RNA polymerase beyond a certain "critical mass" would provoke from a cell a crescendo recitation of the portion of its genetic information (including that for RNA polymerase) which has not been forgotten during the history of that cell. Presumably, the ultimate catastrophe would be avoided by an eventual limitation of the supply of nutrients. How can such a process be reversed in the absence of the hormone? One might suppose that the permeability of the cell would once again limit the supply of nutrients until the level of RNA polymerase, because of spontaneous breakdown, fell below the "critical mass." An obvious prediction of such a model is that a minimal dose of a given hormone might elicit an all-or-none response from the target cells as far as protein synthesis is concerned.

Talwar et al. (111) have made the observation that a soluble fraction from the uteri of ovariectomized rats has two extremely interesting properties: (*a*) it inhibits the RNA polymerase of *E. coli*; and (*b*) it contains a macromolecular receptor for estradiol. Furthermore the ability of the extract to inhibit RNA polymerase is neutralized by the addition of estradiol. Ironically, the extracts are derived from the cytoplasmic fraction of the cells, whereas RNA polymerase exerts its function in the nucleus. If it emerges that the two properties mentioned above actually reside in the same macromolecule, and that rat RNA polymerase is inhibitable in the same fashion as is the bacterial polymerase, these findings would constitute a major advance in our understanding of gene action in mammals.

Differentiation—a few general remarks.—It is now a matter of general agreement that, at least in higher plants, development does not entail the physical loss of genetic information [Steward et al. (112)]. It is at least equally certain that the genetic material can be altered in its potential for expression

by its experience in various types of cytoplasm. These alterations are sufficiently stable to persist through many rounds of mitosis, as in the inactivation of all but one of the X chromosomes in mammals [Lyon (113); Davidson, Nitowsky & Childs (114)]; some such changes are even stable through meiosis. The latter phenomenon, termed "paramutation," has important implications in the study of differentiation and has recently been reviewed [Brink (115)]. The reader is referred to a valuable discussion therein of the physiological significance of euchromatin and heterochromatin. Suffice it here to say that Brink distinguishes between two levels of control in higher organisms. The first, with which the review is largely concerned, involves whole chromosomes or large sections of chromosomes, as in the Lyon hypothesis. This type of control is manifested by the degree of interphase coiling or condensation of various sections of a chromosome; quite possibly, the coiling is also correlated with the presence of histones. Brink points out that control at the level of large sections of chromosomes does not seem to have the specificity that would be demanded of systems analogous to repression/induction in microorganisms, and postulates a second, or "gene-specific," level of control which corresponds to the latter. Presumably, the uncoiled state at the first level of control is *sine qua non* for the expression of gene-specific control. Direct histochemical evidence of this has been presented by Littau and coworkers (116).

Because of limitations of space, one of the most actively pursued aspects of control mechanisms must be slighted in this review—namely, the histone-mediated inhibition of transcription. A symposium dealing with the chemistry and biology of histones has recently been published (117). For a current view of this field, the reader is referred to the latter and to the review by Murray in this volume of the *Annual Review of Biochemistry*. Ursprung (118) has noted that nonhistone nuclear proteins may also have a key role in differentiation.

The provocative hypotheses of Monod & Jacob (119) on the mechanism of differentiation are increasingly open to experimental test. How directly are the events of differentiation attributable to a series of cues from the genome? To what extent is the temporal sequence of these cues dependent upon some sort of feedback discourse with the cytoplasm? Recent evidence obtained by Clever (120) through use of actinomycin and puromycin suggests that in the salivary gland chromosomes of *Chironomus tentans* the puff formation which results from the injection of ecdysone is a prerequisite for the ensuing sequence of puffs. Furthermore, the latter sequence appears to be conditional upon protein synthesis following formation of the puffs which are induced by ecdysone.

In apparent conflict with these results are some findings obtained with *C. thummi*. Kroeger (121) has developed techniques for surgically removing various amounts of chromatin and observing the pattern of puffing in the remaining chromatin. He found that each puffing locus behaves as if it were independent of other loci, and he postulates that the control system for

gene action resides in the nucleoplasm and does not depend on gene interactions. Laufer, Nakase & Vandenberg (122), also working with *C. thummi*, have reported that puromycin, in contrast to actinomycin, affected puffing only after prolonged treatment. The effect of a variety of other inhibitors on the action of ecdysone has been examined by Sekeris & Karlson (123).

A relatively tractable cyclic change in metabolism is the circadian rhythm of photosynthesis in *Acetabularia*, for which the question of control by messenger RNA may again be asked. Schweiger, Wallraff & Schweiger (124) found that enucleate fragments maintained this rhythm even during continuous illumination for up to 40 days. Unfortunately, the possibility of circadian fluctuations in RNA synthesis directed by chloroplast DNA cannot be excluded in these experiments. Although the nucleus is not necessary for the persistence of this periodicity, it apparently is involved in setting the phase in the first place (125). Once again, the postulation of a "long-lived" messenger RNA involved in maintaining the periodicity merely begs the question of how this messenger is translated in an orderly and periodic fashion.

A similar dilemma arises in interpreting the role of "morphogenetic substance" in the longer-term development of this alga. For example, Zetsche (126) and Brachet & Denis (127) have found that nucleate fragments containing little "morphogenetic substance" fail to undergo morphogenesis in the presence of small amounts of actinomycin D, whereas the enucleate stalks, which contain much larger amounts, are much less sensitive to inhibition. Spencer & Harris (23) showed that regeneration, after amputation of the cap in *Acetabularia*, is correlated with the appearance of a phosphatase with a pH optimum of 12.0. Both the regeneration of the cap and synthesis of the enzyme occurred normally in enucleate cells. In summary, it seems certain that messenger RNA is involved in development to an important degree but is not sufficient, in itself, to explain all of the complex sequential events of morphogenesis.

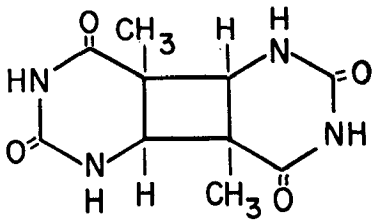
DNA Repair Mechanisms

The study of radiation damage to cells and of recovery from it is a subject with a long history. It has acquired new impetus in recent years with the elucidation of the molecular nature of certain ultraviolet-induced lesions in DNA and with the discovery of bacterial mechanisms which repair this damage. These advances are of great interest not only for the study of mutagenesis but, possibly, also for the investigation of genetic recombination (see below).

Thymine dimers.—A major effect of the irradiation of DNA with ultraviolet in the neighborhood of 260 mμ is the dimerization of thymine. Thymine dimer (I) was identified by Beukers & Berends (128) in ultraviolet-irradiated frozen solutions of thymine. The dimerization also occurs in DNA, where it involves adjacent thymine molecules in the same polynucleotide chain. Steric considerations suggest that in DNA the dimers have structure

(II). Dimerization of thymine molecules in opposite strands of DNA is not detectable in ultraviolet-irradiated phage in the biologically effective dose range (129). Wacker (130) has suggested, however, that such intermolecular cross-links may be formed in growing cells at the time of separation of the two strands. Chemical and biological evidence indicates that thymine dimerization is the major, but not the only, ultraviolet lesion produced in DNA. Smith (131) has detected a number of minor photoproducts of thymine in DNA irradiated *in vivo*. In addition, cytosine and cytosine-thymine dimerization can occur (130, 132). Ionizing radiations, on the other hand, do not produce thymine dimers in solutions of thymine (130), nor is thymine dimerization an important cause of lethality in γ-rayed phage (133). The chemistry and biology of thymine dimers have been reviewed by Wacker (130).

Biological effects of thymine-dimer formation.—A considerable amount of evidence suggests that the lethal effects of ultraviolet are due chiefly to the formation of thymine dimers in DNA (130). Experiments by Setlow, Swenson



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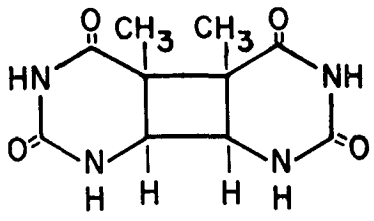

II

& Carrier (134) indicate that thymine dimers block DNA replication *in vivo*. It must be admitted, however, that, although the evidence for lethality of thymine dimers is extensive, it is circumstantial, and some discrepancies—or apparent discrepancies—remain to be explained. The arguments pro and con the importance of thymine dimers in ultraviolet inactivation of bacteriophage have been discussed by Wulff (135). In experiments with phage T4v_1, an ultraviolet-sensitive mutant of T4, Wulff measured thymine-dimer production and lethality as a function of ultraviolet dose at low doses. He found 4.8 dimers per lethal hit; this means that the majority of thymine dimers had no effect on viability. In a similar study with T4*vx*, an even more ultraviolet-sensitive mutant, Sauerbier (136) found 2.4 thymine dimers per lethal hit. Since neither of these mutants has any known dark-repair mechanism for ultraviolet damage, one possible interpretation of the results is that thymine dimerization is not lethal in phage.

However, other explanations are possible as Wulff points out. An unknown repair mechanism may be operating, or there may be a by-pass mechanism which enables the uninjured DNA strand to take over all vital functions in the vicinity of the thymine dimer. It is also possible that some dimers are lethal and others are not, depending on the base sequence or the particu-

lar function of the damaged region. Some findings by Johns et al. (137) may be relevant here. In a study of the photochemistry of the model compound, thymidylyl-(3′→5′)-thymidine (TpT), these investigators detected four chromatographically separable products following ultraviolet irradiation. Two of the photoproducts were identified as intramolecular dimers, designated $\overline{TpT}^1$ and $\overline{TpT}^2$. These compounds were produced in relative yields of 5 to 1. The authors point out that the results of Wulff could be explained if $\overline{TpT}^2$ were the biologically effective dimer and $\overline{TpT}^1$ were relatively innocuous. The problem, thus, has many possible solutions that do not require abandonment of the idea that the lethal effects of ultraviolet are due largely to thymine-dimer formation, but the question is still unresolved.

Photoreactivation.—A variety of repair phenomena has been found in ultraviolet-irradiated bacteria and in ultraviolet-irradiated bacteriophages which are subsequently allowed to infect bacteria. Repair effects are seen on mutational, as well as lethal, ultraviolet damage. Harm (138) has reviewed DNA repair mechanisms in phage.

Until recently, the best known repair mechanism was photoreactivation, discovered by Kelner in 1949 [reviewed by Dulbecco (139)]. This effect consists in the partial elimination of the lethal and mutagenic effects of ultraviolet by light of longer wavelengths (330 to 450 mμ). Rupert (140) showed that photoreactivation depends on an enzyme which can be obtained from yeast and *E. coli*, but not from nonphotoreactivable species such as *Hemophilus*. The enzyme combines with ultraviolet-irradiated, but not with unirradiated, DNA in the dark. The complex dissociates in light of wavelength circa 370 mμ, with simultaneous repair of a fraction of the ultraviolet damage. Wulff & Rupert (141) found that yeast photoreactivating enzyme, in the presence of light, destroys thymine dimers in DNA, presumably by splitting them to thymine. Experiments by Setlow & Setlow (142) with transforming DNA confirm that photoreactivation involves dimer splitting. The DNA was inactivated with large doses of far ultraviolet.[4] It was then reactivated with photoreactivating enzyme and near ultraviolet, followed, in some cases, by additional irradiation with ultraviolet at 239 mμ. The latter wavelength splits thymine dimers nonenzymatically. No additional reactivation by 239 mμ ultraviolet was found in DNA which had been enzymatically photoreactivated, showing that at least some of the photoreactivation was the result of dimer splitting. If irradiation with 239 mμ ultraviolet preceded photoreactivation, an additional reactivation was observed, but this was expected since 239 mμ ultraviolet does not split all of the dimers. In a later study, it was shown by direct analysis that photoreactivated *E. coli* contain fewer thymine dimers than do nonphotoreactivated bacteria (134). It is generally believed that thymine-dimer formation is the most important, although possibly not the only, photoreactivable lesion in DNA.

[4] Far ultraviolet refers to wavelengths below 300 mμ, near ultraviolet to wavelengths between 300 and 400 mμ.

A mutant, designated *phr*$^-$, of *E. coli* B which is nonphotoreactivable and which lacks the photoreactivating enzyme has been found by Harm & Hillebrandt [see Harm (138)]. Using a radiation-resistant derivative of this mutant, Witkin, Sicurella & Bennett (143) showed that photoreversal of ultraviolet-induced mutations to streptomycin resistance, as well as of ultraviolet killing, fails to occur. This result implies that dimerization of thymine can result in mutations, as well as in death. On the other hand, ultraviolet-induced mutations to prototrophy in an arginine-requiring derivative of *phr*$^-$ were photoreversed. In a subsequent paper, Witkin (144) showed that photoreversal of mutations to prototrophy is quantitatively more effective in *phr*$^+$ than in *phr*$^-$ for short exposures to photoreversing light, but with longer exposures there was no significant difference between the strains. It is likely that the mechanism of photoreversal in Witkin's mutants carrying the *phr*$^-$ gene is the same as that involved in photoprotection (see below)—*i.e.*, a light-induced delay in growth and division which allows time for dark-repair processes to act. These findings and others (for the details of which, the papers by Witkin should be consulted) lead to the conclusion that the mutations to prototrophy studied by Witkin differ in some fundamental way from mutations to streptomycin resistance and from ultraviolet killing. Either thymine dimers do not lead to mutations to prototrophy in her system, or, if they do, they differ in some way from the thymine dimers that result in killing or in mutations to streptomycin resistance (145). It may be an interesting coincidence that the mutations to prototrophy studied by Witkin are rarely, if ever, back-mutations, but seem invariably to be the result of suppressor mutations (146).

Photoprotection and liquid-holding recovery.—"Photoprotection" is the term used to describe the reduction in ultraviolet damage which is observed when near ultraviolet is administered to bacteria before exposure to far ultraviolet (147). Harm & Hillebrandt (148) showed that photoprotection and photoreactivation are separate processes by demonstrating that the *phr*$^-$ mutant of *E. coli* B is photoprotected by light administered before (but not after) ultraviolet treatment. Jagger, Wise & Stafford (149) have found that photoprotecting wavelengths (in the vicinity of 338 mμ) cause a delay of growth in *E. coli* B, and they suggest that the essential effect of photoprotection is to give more time for intracellular dark-repair mechanisms to act before DNA replication fixes mutations. This conclusion is supported by kinetic and spectral data and by the fact that *E. coli* B_{s-1}, a mutant that is unable to perform dark-repair of ultraviolet damage (see below), is not photoprotectable.

Jagger and co-workers also investigated the relation between photoprotection and "liquid-holding recovery." The latter is the recovery process which occurs when irradiated cells are held in phosphate buffer for several hours. It was found that the two processes overlap completely—i.e., photoprotected cells show no liquid-holding recovery, and liquid-holding-recovered cells show the same survival as photoprotected ones. Both treatments thus pro-

vide time for the same dark-repair processes to occur. Castellani, Jagger & Setlow (150) have found a similar relation between liquid-holding recovery and photoreactivation; that is, cells given an optimum recovery treatment of one type show no further recovery when subjected to the other treatment. Metzger (151) has found that host-cell reactivation—the term applied to the dark-repair mechanism of bacteria when it operates on ultraviolet-irradiated bacteriophages—repairs the same damage as does photoreactivation. The cumulative meaning of these findings thus seems to be that with respect to lethal ultraviolet effects there are two basic repair processes in bacteria—photoreactivation and dark-repair—and these act on the same kinds of genetic damage.

Dark-repair.—Dark-repair of ultraviolet-induced damage is demonstrable by a number of techniques—liquid-holding recovery, host-cell reactivation, and others. An important advance in our understanding of the mechanism of dark-repair in *E. coli* has been made by Setlow & Carrier (152) and by Boyce & Howard-Flanders (153), who have shown that thymine dimers are not split in dark-repair as they are in photoreactivation, but are excised from DNA and appear in the acid-soluble fraction of cell extracts. Thymine dimers are not found as such in the acid-soluble fraction, but in the form of oligonucleotides. In other words, dimer excision involves cleavage of the sugar-phosphate backbone of DNA. After they have been excised from DNA, thymine dimers are no longer subject to photoreactivation. Both groups of workers employed ultraviolet-sensitive mutants in establishing the effect. Setlow & Carrier used B_{s-1}, a radiation-sensitive mutant of *E. coli* B isolated by Hill (154), while Boyce & Howard-Flanders made use of an ultraviolet sensitive mutant of *E. coli* K-12. Neither mutant is capable of host-cell reactivation, and neither mutant is able to excise thymine dimers from its DNA following ultraviolet irradiation, in contrast to the parental strains from which they were derived.

It is presumed that the gaps left after dimer excision are filled by a repair enzyme that utilizes the undamaged DNA strand as a template. Evidence that the undamaged strand is essential for dark-repair has been obtained by Jansz, Pouwels & van Rotterdam (155) in experiments with phage ØX-174. This virus has single-stranded DNA which, after infection, enters a double-stranded replicative form (156). Both DNA's are infective for spheroplasts of *E. coli*. Jansz and co-workers irradiated both forms with ultraviolet and used them to infect spheroplasts of *E. coli* K-12 and its *hcr*$^-$ (host-cell-reactivation deficient) mutant. It was found that survival of irradiated single-stranded DNA was the same in both hosts, whereas survival of double-stranded DNA was higher in *hcr*$^+$ spheroplasts. It was further shown that double-stranded DNA acquired the ultraviolet sensitivity of the single-stranded form, if it was converted (by heating) to single-stranded DNA after ultraviolet irradiation. These results show that ultraviolet-induced lesions in single-stranded DNA are not reparable, whereas those in double-stranded DNA are, as would be expected if the second strand has a template function.

Pettijohn & Hanawalt (157) have obtained additional evidence for repair-replication in ultraviolet-treated bacteria by allowing them to recover in the presence of the thymine analogue, 5-bromouracil. Density-gradient centrifugation of the DNA showed incorporation of the analogue at random positions along single strands. This is consistent with thymine-dimer excision from single strands, followed by repair.

Rörsch, van der Kamp & Adema (158) have obtained dark-reactivation of ultraviolet-irradiated ØX-174 DNA (double-stranded form), *in vitro*, with an extract from *Micrococcus lysodeikticus*. The activity of the DNA was assayed by measuring phage production in spheroplasts of the *hcr*⁻ mutant of *E. coli* K-12. Use of the mutant made it possible to separate *in vitro* repair from host-cell reactivation. From its thermolability, the repair reaction was judged to be enzymatic. It seems likely that only the excision step occurred in the extract, with reconstruction of the damaged strand taking place intracellularly. A similar result has been reported by Elder & Beers (159) with transforming DNA from *Hemophilus* and an extract from *M. lysodeiklicus*. In an earlier paper, Strauss (160) had reported the presence of an enzyme in extracts of *M. lysodeikticus* which degrades alkylated or ultraviolet-irradiated transforming DNA, but not untreated DNA.

The dark-repair mechanism is inhibited by caffeine and acridine dyes. Lieb (161) showed that caffeine and theophylline produce a tenfold increase in the frequency of prototrophs in ultraviolet-irradiated cultures of a tryptophan-requiring mutant of *E. coli* B/r. The evidence supported the interpretation that the drugs interfere with a dark-repair mechanism that tends to eliminate ultraviolet-induced mutations. In a later paper, Lieb (162) showed that induction of *E. coli* K-12 (λ) by ultraviolet is increased by proflavin and caffeine, presumably through inhibition of dark-repair. The drugs had no effect on photoreactivation of the induction. Further evidence for inhibition of dark reactivation and host-cell reactivation by caffeine has been published by Metzger (163) and by Sauerbier (133).

The inheritance of the dark-repair mechanism is multigenic. Howard-Flanders (164) has identified three genes in *E. coli* K-12 that control thymine-dimer excision. No mutants which lack the ability to perform the reconstruction process that presumably follows dimer excision have been reported to date.

Setlow (165) has discussed possible mechanisms by which the repair process could make mistakes resulting in mutations. That the dark processes are not perfectly effective in restoring ultraviolet-damaged DNA is indicated by the fact that photoreactivation (i.e., dimer splitting) always yields a higher percentage of survivors than does dark-repair. Setlow suggests that the reason for this may be that the dark processes make a certain fraction of mistakes. Not all workers are convinced that premutational damage (i.e., the damage on which repair mechanisms act) is located in DNA. Jensen & Haas (166) argue that the primary effect of ultraviolet is the production of modified nucleic acid precursors which may subsequently be incorporated

into DNA. For further details, the reader is referred to the papers of Haas and co-workers.

Dark-repair and spontaneous mutations.—It is of interest to know whether the dark-repair mechanism acts on spontaneous, as well as on ultraviolet-induced, mutations, since various lines of evidence indicate that the spontaneous mutation rate is genetically controlled. Years ago, Fries & Kihlman (167) found that caffeine is mutagenic in fungi, and this effect is also found in bacteria (168). This is the result that would be expected if, as indicated above, caffeine inhibits dark-repair and if the latter prevents the appearance of a fraction of the spontaneous mutations. On the other hand, Hill & Simson (169) have shown that *E. coli* B_{s-1}, which lacks the thymine-dimer excision step of the dark-repair mechanism, is normal in its rate of spontaneous mutation to phage T_1 resistance. The mutant shows a significantly lower rate of spontaneous mutation to furacin resistance, but such mutations are not independent of the original mutation in B_{s-1} since it is known that mutations to furacin resistance are always accompanied by increased radiation resistance (169). Available evidence thus suggests that the ability to excise thymine dimers does not influence the spontaneous mutation rate, or else that thymine dimerization cannot lead to mutations to T_1 resistance. It is not excluded, however, that caffeine inhibits the reconstruction step of the dark-repair process and that this step is active in the repair of spontaneous mutations. At the present time, this can only be regarded as a speculation.

Genetic Recombination and Related Phenomena

Of the fundamental genetic phenomena, recombination has proven to be the most refractory to interpretation in molecular terms. The decade that has elapsed since the enunciation of the Watson-Crick structure of DNA has seen much progress in elucidating the molecular mechanisms of gene action, replication, and mutation, but we are still largely in the dark as regards the events that result in the recombination of linked genes. The classical theory of crossing-over postulated breakage of nonsister chromatids at homologous sites, followed by their reunion in reciprocal new combinations. The discovery of nonreciprocal recombination in bacteriophages and fungi necessitated a reconsideration of this theory. The most prominent newer hypothesis has been that of "copy-choice," or "switch-synthesis," according to which, recombination does not require a physical exchange of genetic material, but only a recombining of the genetic information contained in the parental chromosomes. These developments have been critically reviewed by Westergaard (170).

It has been clear for some time that copy-choice could not by itself account completely for recombination in chromosomal organisms, but that some breakage and reunion of strands must also be invoked [Stadler & Towe (171)]. Recent work makes it appear unlikely that the copy-choice mechanism plays a significant role in recombination in phage or in the integration of transforming DNA in bacteria, a process which is formally similar to recom-

bination. On the contrary, breakage and reunion of DNA strands has been found in both cases. Tomizawa & Anraku (172, 173) have investigated the molecular mechanism of recombination in phage T4. The addition of KCN to infected cells inhibited DNA synthesis without inhibiting recombination. In fact, the number of recombinants found in KCN-treated cells was higher than in comparable controls. By labelling the parental phage particles with ^{32}P and bromouracil, it was possible to demonstrate the formation in KCN-treated cells of "joint molecules" of DNA, containing label from both parental DNA's. The authors suggest that the "joint molecules" are heterozygotes, which many genetic studies have shown to be the first products of recombination in bacteriophage.

In a continuation of earlier experiments with phage λ, Meselson (174) has performed a two-factor cross in which both parents were labelled with ^{13}C and ^{15}N. Among the progeny, recombinants were found whose DNA was fully labelled. This can only mean that recombination occurred by breakage and reunion of double-stranded parental DNA molecules. There was some indication that a small fraction—up to 5 or 10 percent—of the recombinant DNA was not parental, but resulted from DNA synthesis accompanying recombination. This observation suggests that a small amount of DNA may be removed and resynthesized in the formation of recombinants, and it leads to a model of the recombination process in which reactions similar to those occurring in dark-repair of ultraviolet damage play a part.

The integration of transforming DNA into the genomes of pneumococcus and of *B. subtilis* has been studied, respectively, by Fox & Allen (175) and Bodmer & Ganesan (176). Although the detailed findings differ in a number of respects, both studies show that transformation involves the physical incorporation of donor DNA into the recipient genome. In both cases, it appears that only one strand of donor DNA is incorporated. Since the replaced strand in the receptor DNA must be removed and new covalent linkages formed with the donor strand, the possible relationship to the dark-repair processes described above is clear.

The molecular details of recombination in higher organisms are not known, but recent theoretical treatments by Whitehouse (177, 178) and Holliday (179) lead to models which account for most of the genetic findings in a unitary way—i.e., without invoking separate processes for reciprocal and nonreciprocal recombination. The Whitehouse and Holliday hypotheses, which are similar in principle, postulate, as the first step in recombination, breakage and reunion of single DNA strands in paired double-stranded chromatids. Correction of the resulting heterozygosity by enzymes which recognize and repair faulty base pairing would explain nonreciprocal recombination.

Consonant with the view that recombination involves breaking and repair is the finding by Wimber & Prensky (180) that a small, but consistent, amount of DNA synthesis occurs during the first meiotic prophase in *Triturus* males. Previous workers had failed to detect any synthesis of DNA during

meiotic prophase, and this has been a major stumbling block in the development of a satisfactory theory of crossing over.

In view of the possibility that DNA repair enzymes may be involved in recombination, it is of interest to note that Holliday (181) finds that caffeine induces mitotic crossing-over in *Ustilago*. This result contradicts the simple expectation based on the effect of caffeine on bacterial repair systems (see above).

Nonchromosomal Genes

Classical genetic studies revealed the existence not only of the chromosomal system of inheritance, but also of a category of genes whose mode of transmission indicates that they are located in the cytoplasm. Some of these nonchromosomal genes have been identified with infective particles that are indistinguishable from viruses. Others, however, are normal cellular constituents which appear to be associated with the chloroplasts and mitochondria. Still others have not been definitely associated with any subcellular organelles. One of the major problems in this field is the chemical identity of the cytoplasmic genes. Another, and much debated, question concerns the amount of genetic information carried in the nonchromosomal system, as compared with that in the nucleus. The answer to the first question may make it possible to settle the second. Considerable interest in these and related questions is evident in the current literature. Several reviews and monographs on cytoplasmic inheritance have recently appeared (182–185).

Chloroplasts.—DNA, whose presence in chloroplasts had been suggested by earlier investigations, has now been identified as a constituent of chloroplasts in algae and in higher plants. A major difficulty is the necessity of excluding contamination by nuclear DNA. This difficulty has been overcome in various ways. In the unicellular green alga, *Acetabularia*, the nucleus is located in the base of the stalk and can be removed surgically before isolating the chloroplasts. Gibor & Izawa (186) used this method to enucleate *Acetabularia* cells which, in addition, had been grown axenically. Chloroplasts were isolated by differential centrifugation and their DNA determined before and after DNase treatment. The DNA content per plastid was estimated at 10^{-16} g. In a related study, Schweiger & Berger (187) measured the incorporation of ^{14}C-uracil into acid-insoluble material by chloroplasts from enucleated *Acetabularia*. The incorporation was light-dependent and was inhibited by actinomycin D and DNase. In an experiment in which all four ^{14}C-labelled nucleoside triphosphates were used, the acid-insoluble material was isolated, hydrolyzed in alkali, and the nucleoside 2′,3′-phosphates identified in the hydrolysate.

Autoradiography has been utilized by various authors to demonstrate nucleic acid and protein synthesis in chloroplasts. In this way, Wollgiehn & Mothes (188) have shown incorporation of tritiated thymidine into the chloroplasts of young, but not old, leaves of *Nicotiana*. The fixed radioactivity could be removed by DNase treatment, a control which is essential in

view of the fact that thymidine can be used for RNA synthesis by some species (189). Olszewska & Mikulska (190) have carried out an autoradiographic study of the incorporation of tritiated thymidine, uridine, and phenylalanine into the chloroplasts of *Clivia* and *Bilbergia*. Incorporation of the nucleosides was most rapid in mature chloroplasts, whereas phenylalanine was taken up most readily by proplastids. Both DNA and RNA were synthesized, as judged by the effects of DNase and RNase on the incorporation.

Chloroplast DNA has a different base composition from the nuclear DNA of the same species, as shown by chemical analysis of the isolated DNA's (191) and by density-gradient centrifugation (192–195) (Density-gradient centrifugation is being increasingly used to separate nuclear from cytoplasmic DNA). Chun, Vaughan & Rich (192) found that DNA from spinach and beet leaves shows three density peaks in CsCl density-gradient centrifugation. Nuclear preparations showed only the major peak, whereas chloroplast preparations were enriched tenfold to 30-fold in both minor peaks. One of the minor peaks may actually be associated with mitochondria, which were present in the chloroplast preparations. Only one minor peak was found in DNA from the algae *Chlamydomonas* and *Chlorella*.

Sager & Ishida (193) have shown that the satellite (i.e., the minor peak) of *Chlamydomonas* is chloroplast DNA. They estimated the DNA content at 10^{-14} g per plastid of which 25 to 40 percent was satellite DNA. It is not yet clear whether chloroplasts actually produce both density classes of DNA or whether only the satellite DNA is real chloroplast DNA, the rest being a nuclear DNA contaminant. Leff & Mandel (194) have found a major and a satellite peak in density-gradient centrifugation of DNA from *Euglena*. An aplastidic mutant lacked the satellite. The same authors found two minor peaks in *Chlamydomonas* DNA, one of which is missing in *Polytoma*, a naturally occurring aplastidic chlamydomonad. Ray & Hanawalt (195) report that the satellite DNA of *Euglena* contains at least two distinct size classes: a larger one of mol wt $20 \text{ to } 40 \times 10^6$ and a smaller one of mol wt 2.6×10^6.

Chloroplasts appear to be the site of intensive RNA and protein synthesis. According to Rhodes & Yemm (196), from 70 to 80 percent of the protein of green leaves is contained in the chloroplasts, and, in barley seedlings, 20 to 30 percent of the RNA as well. These authors find that the plastids of barley seedlings become labelled with ^{15}N (supplied as nitrate or ammonium) faster than the microsomes. Kirk (197) has investigated the synthesis of RNA in chloroplasts isolated from broad-bean leaves. ^{14}C-labelled ATP was incorporated into an acid- and ethanol-insoluble, RNase-solubilized form by the chloroplasts. CTP, GTP, and UTP were all required. DNase and actinomycin D inhibited the reaction, which was not light-dependent. The incorporation was five to twelve times faster than that obtained with a nuclear fraction. This, together with the greater sensitivity of the chloroplast system to inhibition, suggests, but does not prove, that nuclear contamination was not responsible for the incorporation.

App & Jagendorf (198) incubated isolated spinach chloroplasts with ^{14}C-amino acids from an algal hydrolysate and showed incorporation of radioactivity into the acid-insoluble fraction. The incorporation was stimulated by light and was inhibited by chloramphenicol, but not by RNase. Ribosomes were then extracted from the chloroplasts with deoxycholate, and these were found to incorporate amino acids in a process that was sensitive to RNase and chloramphenicol. The rate of incorporation by the ribosomes was low, and the authors make no claim to have demonstrated that ribosomes are responsible for amino acid incorporation by chloroplasts. Somewhat different results were obtained by Eisenstadt & Brawerman (199), working with chloroplasts and chloroplast ribosomes from *Euglena*. As with spinach chloroplasts, amino acids were incorporated into protein, but the process was not light-stimulated, nor was it sensitive to chloramphenicol. It was inhibited by RNase and puromycin. Polyuridylic acid stimulated the incorporation of phenylalanine. Ribosomes obtained by deoxycholate treatment of *Euglena* chloroplasts incorporated leucine at a high rate (about one-half the rate of intact chloroplasts) when supplemented with a fraction designated as "DNA-like RNA." The latter appears to be a form of messenger RNA. Chloroplast ribosomes differed from those in the cytoplasm in respect to the base composition of their RNA, their sedimentation behavior, and their response to "DNA-like RNA."

In contrast to the foregoing results are those of Ruppel (200), who worked with chloroplasts from adult leaves of *Allium* and *Antirrhinum*. The RNA of the chloroplasts was found to be virtually identical in base composition with that of ribosomal RNA from the same species. What is more, no thymine could be detected in hydrolysates of the chloroplasts. Whether the same results would be found with young leaves remains to be seen.

Mitochondria.—Compelling genetic evidence exists for the conclusion that some of the mitochondrial cytochromes are determined by nonchromosomal genes. It does not necessarily follow, however, that the mitochondria themselves are so determined. The origin of mitochondria is a separate problem that requires investigation in its own right.

Evidence for increase of mitochondria by growth and division of preexisting mitochondria has been obtained by Luck (201, 202) in ingenious experiments with *Neurospora*. A choline-requiring mutant was grown with tritiated choline and then transferred to a medium containing a large excess of unlabelled choline. Samples were removed at various times during exponential growth in the unlabelled medium, and mitochondria were isolated. Grain counts over individual mitochondria, made by radioautography, showed that the radioactivity (incorporated into phospholipid) was randomly distributed among the mitochondria. This result is inconsistent with *de novo* synthesis of mitochondria, which would predict two unequally labelled populations of mitochondria. It is most readily explained by the assumption that mitochondria multiply by fission. In a subsequent paper, Luck & Reich (203) showed that *Neurospora* mitochondria contain DNA

and, in addition, RNA-polymerase activity. The mitochondrial DNA comprised less than 1 percent of the total cellular DNA; its amount was approximately 0.7 μg per mg of mitochondrial protein. It differed from nuclear DNA in buoyant density, as do chloroplast DNA's (see above). Electron micrographs showed it to be linear and double-stranded. *Neurospora* mitochondrial preparations incorporate labelled GTP into acid-insoluble form; the simultaneous presence of ATP, CTP, and UTP is required. The incorporation is inhibited by actinomycin D, but not by RNase or DNase. Insensitivity to inhibition by enzymes suggests that the polymerase is protected by the mitochondrial membranes.

DNA has also been found, in association with yeast mitochondria, by Schatz, Haslbrunner & Tuppy (204). The authors isolated mitochondria by flotation in Urografin (N,N′-diacetyl-3,5-diamino-2,4,6-triiodobenzoate). Sucrose-gradient centrifugation proved unsatisfactory, because, although the mitochondria formed a well-defined band, soluble DNA was found diffusely distributed throughout the gradient. This was not the case when the flotation method was used. The mitochondria contained DNA in the amount of 1.1 to 4.3 μg per mg of protein.

Wintersberger (205) has studied RNA synthesis in rat-liver mitochondria isolated by the flotation method. Tritiated uridine was incorporated into acid-insoluble material in a reaction that was inhibited by actinomycin. The incorporated counts were solubilized by RNase, but the same effect was found in controls without RNase; this led the author to the finding that trichloroacetic acid-precipitated mitochondria have a small, but measurable, RNase activity.

Autoradiographic studies on slime-mold mitochondria by Guttes & Guttes (206) showed incorporation of tritiated thymidine into an acid- and RNase-stable DNase-unstable, form. A similar study by Wolstenholme & Plaut (207), on *Amoeba*, showed thymidine incorporation into bodies approximately 0.5 μ in diameter which the authors state are not mitochondria.

Morphological studies by Bell & Mühlethaler (208) on egg cells of the fern, *Pteridium*, are not reconcilable with self-duplication of mitochondria. Electron micrographs show that the mitochondria degenerate in the very young egg, but they reappear in the cytoplasm at a later stage. The authors' observations lead them to propose that the mitochondria are regenerated from the nucleus, since the latter was seen to be forming conspicuous evaginations during the time of mitochondrial reappearance. This hypothesis is based solely on morphological criteria; the authors emphasize that they have no chemical evidence that identifies the bodies budded from the nucleus as incipient mitochondria. It should be noted, however, that these observations are not unique; the original paper should be consulted for references to similar observations by other investigators. In another paper by the same authors (209), it is shown by autoradiography that ^{3}H-thymidine is incorporated into the mitochondria and proplastids of *Pteridium* egg cells.

An electron microscope study of apparent *de novo* synthesis of mito-

chondria in yeast cells has been carried out by Wallace & Linnane (210). Anaerobically grown yeast is devoid of mitochondria, and, on aeration, mitochondria are formed in a short time. The anaerobically grown cells show a featureless cytoplasm. Cytoplasmic vesicles, clustered near the nucleus, were seen within 15 min after admitting oxygen. According to the authors, these vesicles become the mitochondria. There is evidence, however, of promitochondria in anaerobic yeast [Yotsuyanagi, cited by Gibor & Granick (182)].

Conclusions.—Considerable evidence favors the conclusion that chloroplasts and mitochondria are self-duplicating. It appears that formation of these bodies can occur either by fission of pre-existing plastids and mitochondria or by growth and development of undifferentiated proplastids and promitochondria. Both chloroplasts and mitochondria contain DNA which differs in base composition from nuclear DNA. DNA synthesis and a DNA-dependent formation of RNA take place in the particles. Although not rigorously proven, it is very probable that this DNA is responsible for the extrachromosomal inheritance which has long been associated with chloroplasts and mitochondria.

Chloroplast DNA accounts for 1 to 5 percent of the total DNA in the species that have been examined. Gibor & Granick (182) estimate the DNA content per plastid at 10^{-15} to 10^{-16} g for many algae and higher plants. An exception is *Chlamydomonas*, where some 3 percent—or 10^{-14} g—of the cellular DNA is contained in the single chloroplast (193). Gibor & Granick suggest that the *Chlamydomonas* plastid may be polyploid.

Available data permit only a rough estimate of the DNA content of mitochondria. Electron microscope pictures show a minimum of 13×10^{6} daltons, or 2×10^{-17} g, of DNA per mitochondrion of *Neurospora* (203). An independent order-of-magnitude estimate can be derived from the amount of DNA per mg of mitochondrial protein in *Neurospora* and *Saccharomyces* (see above). The mitochondria of these two ascomycetes have similar dimensions (211, 212). Taking their average volume as 0.1 μ^3, density 1.2, and protein content 70 percent by dry weight (213), one obtains an estimate of 2 to 18 $\times10^{-17}$ g as the DNA content per mitochondrion. This figure suggests that mitochondria contain less DNA then do chloroplasts. The DNA content of the haploid *Neurospora* nucleus is 4.6×10^{-14} g (214), and that of yeast is 2.5 $\times10^{-14}$ g (215). Thus, the genetic information contained in a mitochondrion may be of the order of 0.05 to 0.5 percent of that in the nucleus.

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